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Conversion of Chlorinated Propanes by *Methylosinus trichosporium* OB3b Expressing Soluble Methane Monooxygenase

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Conversion of Chlorinated Propanes by *Methylosinus trichosporium* OB3b Expressing Soluble Methane Monooxygenase

Chlorinated propanes are important pollutants which may show persistent behavior in the environment. The biotransformation of 1-chloropropane, 1,2-dichloropropane, 1,3-dichloropropane and 1,2,3-trichloropropane was studied using resting cell suspensions of *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. The transformation followed first-order kinetics. The rate constants were in the order of 1-chloropropane > 1,3-dichloropropane > 1,2-dichloropropane > 1,2,3-trichloropropane, and varied from 0.07 to 1.03 ml min^{-1} mg of cells^{-1} for 1,2,3-trichloropropane and 1-chloropropane, respectively. Turnover-dependent inactivation occurred for all of the tested chloropropanes. The inactivation constants were lower for 1-chloropropane and 1,2-dichloropropane than for 1,2,3-trichloropropane and 1,3-dichloropropane. Not all chloride was released during cometabolic transformation of the chlorinated propanes and production of monochlorinated- and dichlorinated propanols was found by gas chromatography. The reaction pathway of 1,2,3-trichloropropane conversion was studied by mass spectrometric analysis of products formed in $^{2}H_2O$, which indicated that 1,2,3-trichloropropane was initially oxidized to 2,3-dichloropropionaldehyde and 1,3-dichloroacetone, depending on whether oxygen insertion occurred on the C3- or C2 carbon of 1,2,3-trichloropropane, followed by reduction to the corresponding propanols. The results show that chloropropanes are susceptible to cometabolic oxidation by methanotrophs, but that the transformation kinetics are worse than with cometabolic conversion of trichloroethylene.

Industrial spillage and agricultural usage are the main causes of environmental pollution with chlorinated propanes. These compounds are used as solvents, soil fumigants and intermediates in chemical synthesis. During the synthesis of epichlorohydrin, 1,2,3-trichloropropane is formed as a by-product. Commercial preparations of the nematocide 1,3-dichloropropene, which is used on potato crops, often contain 1,2-dichloropropane. Chloropropanes are suspected carcinogens which frequently occur as contaminants of groundwater and are poorly degraded in the environment and in biological treatment systems. Microbial growth on chlorinated propanes has only been well documented for 1-chloropropane and 1,3-dichloropropane (13), but not for 1,2-dichloropropane and 1,2,3-trichloropropane. However, cometabolic degradation has been demonstrated for 1-chloropropane and 1,2-dichloropropane (17, 20). Recently anaerobic conversion has been described for 1,2-dichloropropane (14). Higher chlorinated compounds such as 1,2,3-trichloropropane are in general very resistant to biodegradation, and this contributes to their persistence in the environment.

In the past decade research has shown that methanotrophs can cometabolically degrade halogenated aliphatic hydrocarbons as a result of the broad substrate range of their methane monooxygenase (17). The methanotroph *Methylosinus trichosporium* OB3b expresses under copper limitation a soluble methane monooxygenase (sMMO). This enzyme catalyses a wide range of oxidation reactions, including the hydroxylation of alkanes, epoxidation of alkenes, and oxidation of ethers, halogenated alkanes and alkenes, and

aromatic compounds (9, 17, 20, 22). The sMMO of this organism converts halogenated aliphatics via different reaction mechanisms, depending on the compound. Mono- and polyhalomethanes were found to be converted by insertion of oxygen into the carbon-hydrogen bond (2). A sequential oxidative and reductive pathway has been suggested for the conversion of vinyl chloride, leading via chloroethylene oxide to glycolic acid, ethylene glycol and chloroacetic acid (6). Direct insertion of oxygen into the carbon-halogen bond has been proposed for 1,2-dichloroethane, chloroacetic acid and chloroacetamide (5, 18). Similar reaction mechanisms have been proposed for cytochrome P450 (4, 7).

Little is known about the possibility to degrade chlorinated propanes by methanotrophs and the mechanism by which they are converted. The purpose of this work was to determine the degradation kinetics of the cometabolic conversion of chlorinated propanes by *M. trichosporium* OB3b. The transformation products were analyzed and the reaction pathway of 1,2,3-trichloropropane was investigated in more detail.

Results

Degradation kinetics of chlorinated propanes. Cell suspensions of *M. trichosporium* OB3b expressing the soluble methane monooxygenase (sMMO) were used for degradation experiments. The cometabolic conversion of four different chlorinated propanes was followed by on-line gas chromatography. At the substrate concentration used (50 μM), the transformation of all compounds followed first-order kinetics. Substrate depletion curves for 1,2-dichloropropane and the first-order fit are shown in Fig. 1A. The first-order rate constants (k_1) obtained after the first addition of the substrate decreased in the order of 1-chloropropane, 1,3-dichloropropane, 1,2-dichloropropane, and 1,2,3-trichloropropane (Table 1).

Table 1. First-order rate constants and inactivation constants for the degradation of chlorinated propanes by *M. trichosporium* OB3b cells expressing sMMO.

Substrate	k_1 (ml·mg cells^{-1}·min^{-1})	C_i (mg cells inactivated·μmole substrate converted^{-1})
1-chloropropane	1.03	0.68
1,2-dichloropropane	0.36	0.67
1,3-dichloropropane	0.58	1.60
1,2,3-trichloropropane	0.07	1.11
trichloroethene[1]	3.10	0.30

[1] van Hylckama Vlieg *et al*., 1996

Cometabolic conversion of chlorinated hydrocarbons may result in turnover-dependent inactivation of the sMMO (11, 16). The inactivation caused by conversion of chlorinated propanes was tested by monitoring substrate depletion rates after repeated additions of these compounds to a concentration of 50 μM.

The repeated addition of 1,2-dichloropropane caused a lower degradation rate after each subsequent addition (Fig. 1A, B). No decrease in the degradation rate occurred with a control culture to which the halogenated substrate was added for the first time after 200 min. Similar depletion curves were determined for the other chloropropanes. The relative first-order rate constants were plotted versus the amount of chloropropane converted (Fig. 1B), and from this the inactivation constants were determined (Table 1). Similar inactivation constants were observed for 1-chloropropane and 1,2-dichloropropane. Both 1,2,3-trichloropropane and 1,3-dichloropropane showed higher inactivation constants, and the degree of inactivation per amount of substrate converted decreased when the cells became less active. This may be due to inactivation being caused by accumulation of unstable reactive products, which would reach lower levels if their rate of formation decreases after each subsequent pulse. All chloropropanes were more toxic than trichloroethylene, for which conversion-mediated inactivation is well established (16).

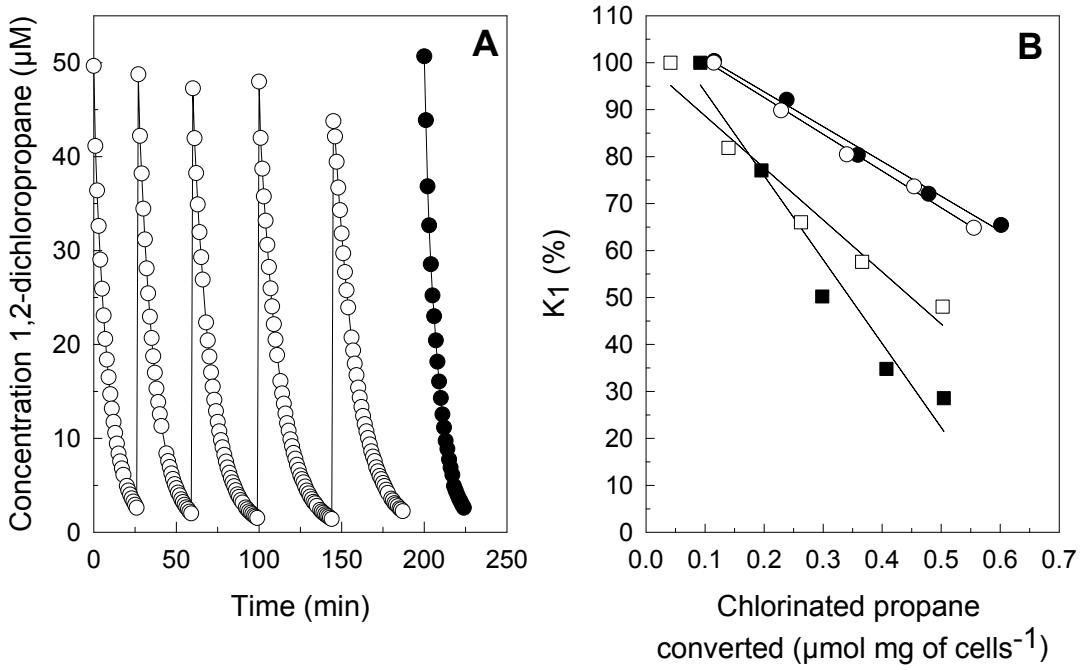

Fig. 1. Effect of repeated additions of chloropropanes on the degradation rate *M. trichosporium* OB3b cells. A) Repeated degradation of 1,2-dichloropropane. As a control, degradation was also measured with cells to which 1,2-dichloropropane was added for the first time after 200 min (•). B) Effect of the amount of chlorinated propane converted on the first-order rate constant expressed as a percentage of the initial rate constant. Compounds: (•), 1-chloropropane; (○), 1,2-dichloropropane; (■), 1,3-dichloropropane; (□), 1,2,3-trichloropropane.

Analysis of products of chlorinated propane transformation. Various intermediates formed during conversion of chlorinated propanes by *M. trichosporium* OB3b were identified by GC analysis of ether extracts of the incubation mixtures. Chlorinated propanols were found as degradation products of 1-chloropropane, 1,2-dichloropropane and 1,2,3-trichloropropane (Table 2). During conversion of 1-chloropropane, 67% of the organic chlorine was liberated and 1-chloro-2-propanol was formed in low amounts.

Table 2. Products formed during the conversion of chlorinated propanes by *M. trichosporium* OB3b.

Substrate	Substrate converted (μM)	Products	Product concentration (μM)	Product yield (% of substrate converted)
1-chloropropane	246	1-chloro-2-propanol	9	4
		chloride	164	67
1,2-dichloropropane	242	1-chloro-2-propanol	26	11
		2-chloro-1-propanol	53	22
		2,3-dichloropropanol	138	57
		chloride	100	21
1,3-dichloropropane	243	chloride	338	70
1,2,3-trichloropropane	219	2-chloro-1-propanol	145	66
		1,3-dichloro-2-propanol	7	3
		2,3-dichloro-1-propanol	24	11
		chloride	410	62

In order to determine the primary products of chloropropane transformation, we tested whether additional alcohols were produced if further degradation was inhibited with cyclopropane. Cyclopropane and cyclopropane-derived compounds are irreversible covalent inhibitors of the methanol dehydrogenase, which oxidizes several primary alcohols (10, 19, 24). For these experiments, 20 ml of cells (0.3 mg cells (dry weight)/ml) freshly taken from a fermentor were incubated at 30°C for 2 h with 1-chloropropane or 3-chloro-1-propanol with or without pretreatment with cyclopropane (1 mM). During 1-chloropropane conversion low amounts of 1-chloro-2-propanol were formed both with cyclopropane-treated cells and with untreated cells. Incubation of cyclopropane treated cells with 1-chloropropane (0.5 mM) yielded mainly 3-chloro-1-propanol (0.3 mM) besides 1-chloro-2-propanol (0.024 mM). With 3-chloro-1-propanol (0.5 mM) complete degradation was observed only for non-treated cells and up to 85% of the organic chlorine was released as inorganic chloride. Using cyclopropane-treated cells, 67% of the added 3-chloro-1-propanol was left and 17% of the organic chlorine was released as chloride. These results indicate that 1-chloropropane was mainly converted to 3-chloro-1-propanol, which was rapidly further transformed and dehalogenated by a route which was slowed down in the presence of cyclopropane,

suggesting that the methanol dehydrogenase was involved in the conversion of 3-chloro-1-propanol.

Accumulation of 2,3-dichloro-1-propanol, 1-chloro-2-propanol and 2-chloro-1-propanol was observed during transformation of 1,2-dichloropropane by resting cells of *M. trichosporium* OB3b. The conversion of 1,2-dichloropropane in a batch incubation was followed in time by GC analysis to identify early intermediates (Fig. 2). The first products of 1,2-dichloropropane conversion were 2,3-dichloro-1-propanol and chloroacetone. As chloroacetone was degraded a concomitant increase of 1-chloro-2-propanol was observed, indicating a reduction.

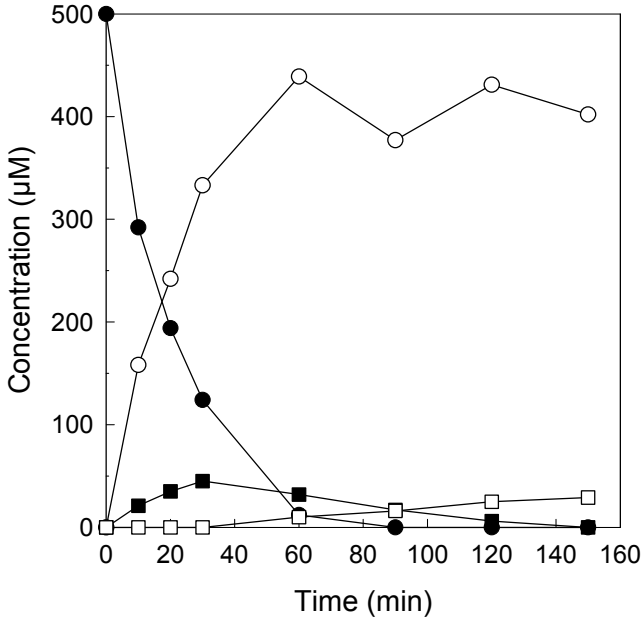

Fig. 2. Conversion of 1,2-dichloropropane (0.5 mM) by *M. trichosporium* OB3b. The cell concentration was 0.82 mg/ml. Symbols: (●), 1,2-dichloropropane; (○), 2,3-dichloro-1-propanol; (■), chloroacetone; (□), 1-chloro-2-propanol.

In a separate batch experiment cells (0.7 mg (dry weight)/ml) were incubated with 0.6 mM 2,3-dichloro-1-propanol. After 65 h 420 µM 2,3-dichloro-1-propanol was left and 150 µM 2-chloro-1-propanol and 200 µM chloride were produced. No degradation of 2,3-dichloro-1-propanol was observed in a control incubation with heat-killed cells. These results indicate that reductive hydrogenolysis of 2,3-dichloro-1-propanol did indeed occur.

After conversion of 1,3-dichloropropane by *M. trichosporium* OB3b, no propane derivatives could be detected. At the end of the experiment, most of the chlorine added as 1,3-dichloropropane was present as inorganic chloride. Considering the high inactivation constant found for this compound, toxic products must be formed during conversion.

Low amounts of 2,3-dichloro-1-propanol and 1,3-dichloro-2-propanol were formed during conversion of 1,2,3-trichloropropane, and 62% of the organic chlorine was liberated as

inorganic chloride. It was found that 2-chloro-1-propanol was the main product of 1,2,3-trichloropropane conversion, again indicating reductive dechlorination.

Reaction pathway of 1,2,3-trichloropropane conversion. The soluble methane monooxygenase of *M. trichosporium* OB3b inserts oxygen into C-H bonds. With 1,2,3-trichloropropane this would lead to the formation of 2,3-dichloropropionaldehyde or 1,3-dichloroacetone, depending on whether oxygen insertion takes place on the C1 or C2 carbon (Fig. 3).

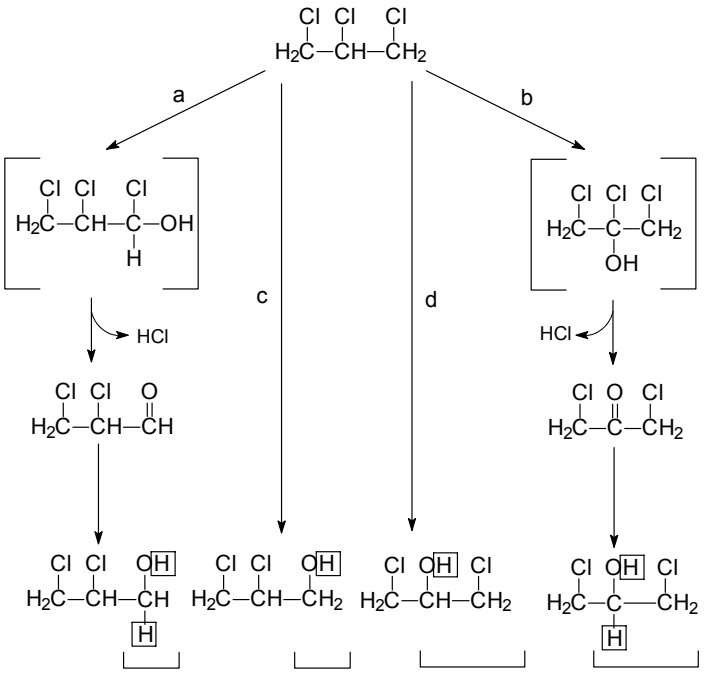

Fig. 3 Possible conversion routes of 1,2,3-trichloropropane by *M. trichosporium* OB3b. The relevant fragments (for 2,3-dichloro-1-propanol: *m/z* 31, for 1,3-dichloro-2-propanol: *m/z* 79, 81) for differentiation between route a, b and c, d are underlined. The formation of dichlorinated propanols could occur via reduction of the aldehyde or ketone (routes a and b) or via direct insertion of oxygen into the carbon-halogen bond of 1,2,3-trichloropropane (route c and d). The introduced hydrogens are indicated in boxes.

The observation that monochlorinated and dichlorinated propanols were formed during conversion of 1,2-dichloropropane and 1,2,3-trichloropropane, respectively, implies additional reaction pathways. The formation of 1,3-dichloro-2-propanol and 2,3-dichloro-1-propanol from 1,2,3-trichloropropane could occur via reduction of 1,3-dichloroacetone or 2,3-dichloropropionaldehyde, respectively (Fig. 3, routes a and b). Reduction of an aldhyde function by *M. trichosporium* OB3b has been demonstrated for trichloroacetaldehyde (chloral hydrate), which was partly reduced to trichloroethanol (15, 17). Other explanations for the formation of dichloropropanols from 1,2,3-trichloropropane would be insertion of oxygen into the C-Cl bond (Fig. 3, routes c and d), or substitutive displacement of the halogen, as has been proposed for 1,2-dichloroethane, chloroacetic acid and chloroacetamide (5, 18). Upon reduction of the aldehyde or ketone two new hydrogen atoms would be introduced (Fig. 3, routes a and b), which must be derived from formate via hydride transfer with NADH or from the solvent via protons and electron transfer. Only one hydrogen would be introduced by direct substitution (routes c and d). In both cases, one hydrogen is on the hydroxyl group and can exchange with solvent. The difference between

these routes is that an additional carbon-bound deuterium should be introduced either with deuterated formate or with deuterium oxide if reduction is involved in the formation of the alcohol. To distinguish between these possibilities, we conducted experiments in which 1,2,3-trichloropropane was converted in the presence or absence of deuterium oxide and deuterated sodium formate, and the number of introduced hydrogens was determined by GC-MS analysis of the products (Table 3).

Table 3 Incorporation ^{2}H during conversion of 1,2,3-trichloropropane (TCP) by whole cells of *M. trichosporium* OB3b. Cells were incubated in buffers containing $^{2}H_2O$ as solvent or Na-d_1-formate. The dichloropropanols formed were analyzed for deuterium content.

Substrate	Buffer	Relative abundances of products							
		2,3-Dichloro-1-propanol			1,3-Dichloro-2-propanol				
		m/z 31	*m/z* 32	*m/z* 33	*m/z* 79	*m/z* 80	*m/z* 81	*m/z* 82	*m/z* 83
Reference	H_2O[a]	100	0	0	100	3	32	0.6	0.03
	$^{2}H_2O$[a]	100	114	0	100	178	36	58	1.5
TCP	$^{2}HCOONa$[b]	100	0	0	100	0	32	0	0
	$^{2}H_2O$[c]	100	443	84	100	179	160	100	42

[a] 2,3-dichloro-1-propanol or 1,3-dichloro-2-propanol dissolved in H_2O or $^{2}H_2O$ without cells.
[b] Deuterated sodium formate was used as source of reducing equivalents.
[c] Freeze dried buffer dissolved in deuterium oxide.

The major fragments found for commercial 2,3-dichloro-1-propanol were: *m/z* 92, 94 (C_3H_5ClO); *m/z* 62, 64 (C_2H_3Cl); *m/z* 31 (CH_3O); and for 1,3-dichloro-2-propanol: *m/z* 79, 81 (C_2H_4ClO); *m/z* 49, 51 (CH_2Cl). The characteristic 3:1 ratio was found for the fragments *m/z* 79, 81 of 1,3-dichloro-2-propanol, due to the natural abundances of ^{35}Cl and ^{37}Cl. The relevant fragments for differentiation between the two reaction pathways were *m/z* 31 (CH_3O) for 2,3-dichloro-1-propanol and *m/z* 79, 81 (C_2H_4ClO) for 1,3-dichloro-2-propanol. Upon incubation of 2,3-dichloro-1-propanol in deuterium oxide without cells, the relevant fragments were: *m/z* 31 (CH_3O); *m/z* 32 (C^2HH_2O); and for 1,3-dichloro-2-propanol: *m/z* 79 ($C_2H_4{}^{35}ClO$); *m/z* 80 ($C_2{}^2HH_3{}^{35}ClO$); *m/z* 81 ($C_2H_4{}^{37}ClO$); *m/z* 82 ($C_2{}^2HH_3{}^{37}ClO$). The shift of one mass unit is explained by chemical exchange of hydrogen and deuterium on the hydroxyl group of the dichloropropanols.

No deuterium was incorporated during conversion of 1,2,3-trichloropropane by *M. trichosporium* OB3b in the presence of deuterated sodium formate, since the mass spectra were similar as for dichloropropanols formed in H_2O. If an NAD-dependent dehydrogenase has the opposite stereospecificity of the NAD-dependent formate dehydrogenase, the hydrogen of NAD^2HH will be transferred to the product instead of the deuterium. However, due to recycling of the NAD/NADH pool via the formate dehydrogenase, both positions on the carbon-4 of NAD will be occupied by a deuterium, which would end up in the product after repeated turnover. During this experiment, 150 µmol of 1,2,3-trichloropropane was converted by 320 mg cells. Assuming a cell volume of 1.7 ml/g cells (dry weight) and an intracellular NAD/NADH concentration of 2 mM (21), it can be calculated that the NAD/NADH pool was recycled 150 times during 1,2,3-trichloropropane conversion.

Incorporation of deuterium did occur during conversion of 1,2,3-trichloropropane in the presence of deuterium oxide. The relative abundances of mass fragments *m*/*z* 32 and 33 for 2,3-dichloro-1-propanol and mass fragments *m*/*z* 81, 82 and 83 for 1,3-dichloro-2-propanol were higher than in the control which contained deuterium oxide but no cells. Furthermore, the two mass fragments *m*/*z* 33 (CD_2HO) for 2,3-dichloro-1-propanol and *m*/*z* 83 ($C_2D_2H_2{}^{37}ClO$) for 1,3-dichloro-2-propanol were only observed in the presence of cells. Thus two deuteriums were incorporated during oxidation of 1,2,3-trichloropropane.

The formation of 1,3-dichloro-2-propanol via reduction of 1,3-dichloroacetone was tested separately by incubating 500 µM 1,3-dichloroacetone at 30°C for two hours. A low amount of 1,3-dichloro-2-propanol (46 µM) was detected. No formation of 1,3-dichloro-2-propanol was observed in a control incubation with heat killed cells. Dichloropropionaldehyde was not tested, because this compound was not available.

The above results indicate that the dichloropropanols were produced during oxidation of 1,2,3-trichloropropane via reduction of 2,3-dichloropropionaldehyde and 1,3-dichloroacetone (Fig. 3, routes a and b).

Discussion

The results of this work show that cells of *M. trichosporium* OB3b expressing the soluble form of the methane monooxygenase can cometabolically transform chlorinated propanes. The first-order rate constants decrease in the order of 1-chloropropane, 1,3-dichloropropane, 1,2-dichloropropane and 1,2,3-trichloropropane. The conversion rates thus decreased with an increasing number of chlorine substituents. The first-order rate constant of 1,2,3-trichloropropane was similar to that previously found for 1,1,1-trichloroethane (0.1 ml min^{-1}/mg of cells). The highest rate constant was observed for 1-chloropropane, which was similar to 1,2-dichloroethane and 1,1-dichloroethylene (1.0 ml min^{-1}/mg of cells) (16). However, compared to an important environmental pollutant such as trichloroethene (TCE), chlorinated propanes are poor substrates.

All chlorinated propanes tested exhibited product toxicity, resulting in a finite transformation capacity (1). The transformation capacities varied from 1.47 µmol/mg for 1-chloropropane and 1,2-dichloropropane to 0.62 µmol/mg for 1,3-dichloropropane. This is low compared to the values found for TCE (2.0 µmol/mg) or 1,2-dichloroethane (10 µmol/mg) under batch incubation conditions (8). The transformation capacities of chlorinated methanes, ethanes and ethenes were proposed to be inversely proportional to their chlorine content (8). Such a clear relation was not found for the chlorinated propanes.

Product analysis showed that oxygen insertion was preferred on the non-substituted carbon since 3-chloro-1-propanol and 2,3-dichoro-1-propanol accumulated during conversion of 1-chloropropane and 1,2-dichloropropane, respectively. The results suggest that the lack of a terminally unsubstituted carbon atom, as for 1,3-dichloropropane and 1,2,3-trichloropropane, increases the toxicity of these compounds. Insertion of oxygen on a chlorine-substituted carbon would yield carbonyl compounds which are probably more reactive than chlorinated propanols (12). Furthermore, hydrogen abstraction, which is

assumed to be the first step in substrate oxidation, leads to a chloroalkyl radical, which may be extremely reactive toward nucleophilic groups in the protein. Experiments with [^{14}C]TCE have shown that the toxicity of TCE is caused by a nonspecific reaction of conversion products with cell components, including the sMMO (11, 16).

The main product of 1-chloropropane conversion, 3-chloro-1-propanol, was only detected with cells in which alcohol dehydrogenase was inhibited by cyclopropane. The relative amounts of the products formed were somewhat different from those found by Shimoda et al. (20). They used cyclopropanol as a selective inhibitor for methanol dehydrogenase and found accumulation, in increasing concentrations, of 3-chloro-1-propanol, 1-propanol and 1-chloro-2-propanol. In our experiments with cyclopropane-treated cells, 3-chloro-1-propanol was the major degradation product, and we detected only low amounts of 1-chloro-2-propanol and no 1-propanol. The difference may be due to variations in cell density, monooxygenase activity, or incubation times.

The amounts of chloride released during the conversion of 1-chloropropane, 1,3-dichloropropane and 1,2,3-trichloropropane were similar. Since 1,2-dichloropropane was mainly converted to 2,3-dichloro-1-propanol, less chloride was released. In the timescale of the experiment (200 min) 2,3-dichloro-1-propanol was almost not converted, although conversion to 2-chloro-1-propanol is possible, probably via reductive hydrogenolysis. Such a reaction has been proposed for the conversion of chloroethylene oxide to ethylene oxide by *M. trichosporium* OB3b (6). The formation of 2-chloro-1-propanol during transformation of 1,2,3-trichloropropane also points to reductive dechlorination occurring with *M. trichosporium* OB3b. In this case 2,3-dichloropropionaldehyde should be an intermediate which is converted to 2-chloro-1-propanol by a combination of carbonyl reduction and reductive hydrogenolysis. These reductive dechlorination reactions occurred in batch cultures in which excess oxygen was present and mass transfer of oxygen was rapid, indicating that reductive dechlorination can occur under aerobic conditions. Whether the methane monooxygenase is involved in this conversion or biomolecules such as a cytochrome or a reduced coenzyme are responsible is not yet known. Reductive dechlorination by mammalian cytochrome P450 has been well established (12).

Another type of reaction which influenced the nature of the products formed from chloropropanes was reduction of carbonyl groups. This reaction explains the formation of 1-chloro-2-propanol from 1,2-dichloropropane, and of both 2-chloro-1-propanol and 1-chloro-2-propanol during conversion of 1,2,3-trichloropropane. For the latter compound it was found that 2,3-dichloro-1-propanol and 1,3-dichloro-2-propanol were formed via reduction of the aldehyde or ketone and that the introduced hydrogens were derived from water. This was further confirmed by using 1,3-dichloroacetone as the substrate, which yielded 1,3-dichloro-2-propanol as the product, while in a heat killed control no 1,3-dichloro-2-propanol was found. So, 1,2,3-trichloropropane was probably mainly converted to 1,3-dichloroacetone and to 2,3-dichloropropionaldehyde. A small fraction of these products was reduced to the corresponding alcohols. The reduction is a minor conversion since only low amounts of dichloropropanols were found and their reduction was

very slow. An initial insertion of oxygen into the C-Cl bond as a major reaction pathway is not likely, because larger amounts of the dichloropropanols would accumulate and the observed incorporation of hydrogen from water into carbon-bound hydrogen of the product would not occur. This is in contrast with conversion of 1,2-dichloroethane for which it was proposed that oxygen was mainly inserted into the carbon-chlorine bond (18).

The results of this work show that *M. trichosporium* OB3b cometabolically converts chlorinated propanes. Compared to other important environmental pollutants, the transformation capacities were low. Bacterial growth has been observed on 1-chloropropane and 1,3-dichloropropane, which of course is preferable over cometabolic conversion. However, for recalcitrant compounds such as 1,2-dichloropropane and 1,2,3-trichloropropane, cometabolic conversion by *M. trichosporium* OB3b may be an alternative. The range of products formed is determined by at least four reactions: carbon hydroxylation, alcohol oxidation, carbonyl reduction, and reductive dechlorination.

Experimental protocols

Organism and growth conditions. *Methylosinus trichosporium* OB3b (NCIB 11131) was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom. The organism was grown continuously in a 3 l fermentor as described by van Hylckama Vlieg *et al.* (1996).

Preparation of suspensions of *M. trichosporium* OB3b. Cells grown in a fermentor were harvested by centrifugation (6000 × g for 5 min at 4° C) and suspended in mineral medium (MMF) supplemented with 20 mM phosphate buffer (pH 6.9) and 20 mM sodium formate. Mineral medium was the same as that described by Janssen *et al*. (1985). Suspensions prepared in this way were used for degradation experiments in batch cultures. These were performed in 25 ml incubations in shake flasks (30° C) at a substrate concentration of 0.5 mM. Samples were taken at different timepoints and analyzed by gas chromatography.

For the deuterium oxide experiments, MMF supplemented with 20 mM phosphate buffer and 20 mM sodium formate was freeze dried, and dissolved in deuterium oxide. The cells were resuspended in this buffer and incubated for 10 min to allow exchange of water. After centrifugation the cells were resuspended in the same buffer and incubated at 30° C with 0.5 mM 1,2,3-trichloropropane for 2 h. Products were extracted with diethylether and concentrated 200-fold by evaporation of the solvent prior to GC-MS analysis.

Treatment with cyclopropane, which can be used to selectively inhibit methanol dehydrogenase, was performed by incubating the cell suspension with 1 mM cyclopropane for 3 - 4 min at 30° C. The excess cyclopropane was removed by air bubbling.

Degradation kinetics. Cometabolic degradation of 1-chloropropane, 1,2-dichloropropane, 1,3-dichloropropane and 1,2,3-trichloropropane by the soluble monooxygenase was determined by on-line analysis of their concentrations in the headspace (van Hylckama Vlieg et al. 1996). The system consisted of a 120 ml double walled glass incubation vessel that was temperature controlled at 30° C. Gas was continuously withdrawn from the headspace and injected back into the magnetically stirred liquid phase with a flow rate of approximately 200 ml/min. With time intervals of one minute, the content of the sample loop (35 µl) was injected into a gas chromatograph. The instrument (Chrompack type CP9001) was equipped with a CPSil-5-CB column (Chrompack; length 25 m, inner diameter 0.53 mm, film thickness 5 µm), and a flame ionization detector (FID). Helium was used as the carrier gas (175 kPa), and the column temperature was 114° C.

To determine the degradation kinetics with 1-chloropropane, 1,2-dichloropropane and 1,3-dichloropropane, 25 ml of cell suspension (0.4 mg cells (dry weight)/ml) was taken directly from the fermentor. For degradation of 1,2,3-trichloropropane the cells were concentrated four-fold. Formate and

phosphate buffer (pH 6.9) were added to a final concentration of 20 mM each. The cell suspension was incubated for 5 min to allow generation of enough reducing power to obtain a maximal initial degradation rate. Assays were then started by adding halogenated substrate from a stock solution in water to a final concentration of 50 μM in the liquid phase.

Analytical methods. Polar halogenated compounds produced during the degradation experiments were analyzed on a Chrompack 438S gas chromatograph equipped with a FID detector and a CPWax-52-CB column (Chrompack; length 25 m, inner diameter 0.25 mm). The carrier gas was nitrogen (50 kPa), and the temperature program was 3 min isothermal at 45 °C followed by an increase to 200 °C at 10 °C/min. Samples (4.5 ml) were extracted with 1.5 ml of diethyl ether containing 0.05 mM 1-bromohexane as the internal standard. The upper layer was analyzed by split injection of 1 μl samples in the gas chromatograph. The identity of the degradation products was confirmed by retention time comparison with authentic standards.

GC-MS analysis was performed on a HP 5890 gas chromatograph with a HP5 capillary column (length 25 m, inner diameter 0.25 mm, film thickness 0.25 μm), connected to a FID and a type 5971 mass selective detector. Helium was used as a carrier gas (0.9 ml/min), and the temperature program was 3 min isothermal at 45° C followed by an increase to 200°C at 10°C/min. Halide levels were determined by the colorimetric method of Bergmann and Sanik (1957).

Chemicals. Halogenated compounds were obtained from Janssen Chimica, Beerse (Belgium) or from Merck, Darmstadt (FRG). $^{2}H_2O$ (99.8% v/v) was purchased from Isotec Inc., Miamisburg OH (USA). Cyclopropane was obtained from Aldrich Chemie, Bornem (Belgium).

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